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On 3 July 2006

TOWNSEND and TOWNSEND and CREW LLP

By: Malinda Wright

PATENT
Attorney Docket No.: 015280-315100US
Client Ref. No.: E-094-1997/0-US-03

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

CHANDRASEKHARAPPA et al.

Application No.: 09/380,337

Filed: March 6, 2000

For: MENI, THE GENE ASSOCIATED WITH MULTIPLE ENDOCRINE NEOPLASIA TYPE 1, MENIN POLYPEPTIDES AND USES THEREOF

Customer No.: 20350

Confirmation No. 2491

Examiner: Ungar, Susan

Technology Center/Art Unit: 1642

SUBSTITUTE APPEAL BRIEF UNDER 37 C.F.R. § 41.37

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Sir:

This substitute brief is pursuant to the Office Communication mailed June 2, 2006 requiring an amended brief and the Examiner Interview of July 3, 2006. Applicants believe no additional fees are due.

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I. REAL PARTY IN INTEREST

THE GOVERNMENT OF THE UNITED STATES OF AMERICA as represented by the Secretary of the Department of Health and Human Services is the assignee of the above-referenced patent application and thus, the real party in interest.

II. RELATED APPEALS AND INTERFERENCES

There are no related appeals, interferences, or judicial proceedings at this time.

III. STATUS OF THE CLAIMS

Claims 1, 3-5, 19-24, 26, 30, 32, 33, 36, and 37 are pending and under examination.

Claims 2, 6-18, 25, 27-29, 31, and 38-42 are cancelled.

Claims 34 and 35 are withdrawn from consideration.

Claims 1, 3-5, 19-24, 26, 30, 32, 33, 36, and 37 are being appealed.

IV. STATUS OF AMENDMENTS

An Amendment under 37 C.F.R. § 1.116 was mailed for filing on July 21, 2005. The Advisory Action mailed December 28, 2005 indicates that the amendment was considered by the Examiner, but not entered.

V. SUMMARY OF CLAIMED SUBJECT MATTER

The claimed invention is based on the discovery that mutations in the gene designated "*MEN1*" are associated with neoplastic disease, *e.g.*, the familial cancer syndrome multiple endocrine neoplasia type I. The *MEN1* gene product is designated "menin".

The subject matter of independent claim 1 relates to an isolated or recombinant nucleic acid encoding menin, where the nucleic acid encodes a protein comprising an amino acid sequence having at least 95% identity to SEQ ID NO:2. Support for this subject matter can be found, *e.g.*, on page 4, lines 4-9, page 10, line 21, and page 14, lines 22-27.

The subject matter of claim 3 relates to an isolated or recombinant nucleic acid encoding a menin protein where the sequence comprises the coding region of SEQ ID NO:1. Support for this subject matter can be found, *e.g.,* in Figure 1 and on page 10, lines 14-18.

The subject matter of claim 4 relates to an isolated or recombinant nucleic acid encoding a menin protein where the sequence comprises SEQ ID NO:3. Support for this subject matter can be found, in Figure 1 and on page 10, lines 14-18.

The subject matter of claim 5 relates to an isolated or recombinant nucleic acid encoding a menin protein having the sequence set forth in SEQ ID NO:2. Support for this subject matter can be found, *e.g.,* in Figure 1 and on page 4, lines 6-9.

The subject matter of independent claim 19 relates to a method for detecting mutations in a human *MEN1* gene or the presence or the absence of a human *MEN1* gene comprising a nucleotide sequence that encodes a human menin as set forth in SEQ ID NO:2. The method comprises contacting the test sample with a first oligonucleotide having a sequence that discriminates between a wild type gene and the missing allele or mutant form; and detecting the formation of a duplex between the gene and the first oligonucleotide sequence. Support for this subject matter can be found, *e.g.,* on page 5, lines 11-19; on page 18, lines 3-19; page 33, lines 3-5 and 18-22; page 34, lines 27-32; on page 35, line 31 bridging to page 36, line 2; and on page 52, lines 5-26; and Figures 2, 3, and 4.

The subject matter of independent claim 24 relates to a kit for detecting the presence or absence of a mutation in a *MEN1* gene comprising a nucleotide sequence encoding a menin polypeptide as set forth in SEQ ID NO:2. Such a kit comprises a container holding a first oligonucleotide sequence that discriminates between the wild type gene and the mutant form; and a container holding a reagent for detecting the formation of a duplex between the gene and the first nucleotide sequence. Support for this subject matter can be found, *e.g.,* on page 6, lines 20-27.

The subject matter of independent claim 30 relates to a transfected cell *in vitro*, wherein the cell comprises a heterologous nucleic acid of claim 1. Support for this subject matter can be found, *e.g.,* on page 25 and page 29, lines 6-7.

The subject matter of claim 32 relates to a transfected cell where the heterologous nucleic acid expressed by the cell comprises a nucleic acid as set forth in SEQ ID NO:1 or SEQ ID NO:3. Support for this subject matter can be found, *e.g.*, on page 6, lines 13-15.

The subject matter of claim 33 relates to a transfected cell where the cell is a human cell. Support for this subject matter can be found, *e.g.*, on, page 6, lines 14-15.

The subject matter of claim 36 relates to an expression cassette comprising a nucleic acid that encodes a menin protein as set forth in current claim 1, where the nucleic acid is operably linked to a promoter. Support for this subject matter can be found, *e.g.*, on page 23, lines 12-20.

The subject matter of claim 37 relates to an expression cassette that is comprised by an expression vector. Support for this subject matter can be found, *e.g.*, on page 21, lines 10-13.

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

Claims 1, 30, 32, 33, 36, and 37 stand rejected under 35 U.S.C. § 112, first paragraph as not enabled.

Claims 1, 3-5, 19-24, 26, 30, 32, 33, 36, and 37 additionally stand rejected under 35 U.S.C. § 112, first paragraph as not enabled.

Claims 3, 4, and 32 additionally stand rejected under 35 U.S.C. § 112, first paragraph as not enabled.

Claims 1, 30, 32, 33, 36, and 37 stand rejected under 35 U.S.C. § 112, first paragraph as lacking adequate written descriptive support in the specification.

Claims 19-24, and 26 stand rejected under 35 U.S.C. § 112, first paragraph as lacking adequate written descriptive support in the specification.

Claims 1, 3-5, 30, 32, 33, 36 and 37 stand rejected under 35 U.S.C. § 102 as anticipated by U.S. Patent No. 4,594,318.

Claim 30 stands rejected under 35 U.S.C. § 112, second paragraph as indefinite.

VII. ARGUMENT

The arguments address the rejections in the order listed in the Final Office Action dated February 24, 2005.

A. Rejection of claims 1, 30, 32, 33, 36, and 37 under 35 U.S.C. § 112

i. Standards for enablement

It is well-settled in the biotechnology art that routine screening of even large numbers of samples is not undue experimentation when a probability of success exists. *In re Wands*, 858 F.2d 731, 8 USPQ2d 1400 (Fed. Cir. 1988). As stated in *Wands*, "enablement is not precluded by the necessity for some experimentation, such as routine screening." *In re Wands*, 858 F.2d at 737, 8 USPQ2d at 1404 (Fed. Cir. 1988). Moreover, as set forth in MPEP § 2164.08, a rejection for undue breadth is inappropriate where a nucleic acid encodes a particular protein sequence and "one of skill could readily determine any one of the claimed embodiments."

In order to establish a *prima facie* case of enablement, the examiner has the initial burden to establish a reasonable basis to question the enablement provided for the claimed invention (*In re Wright*, 999F.2d 1557, 1561-62, 27 USPQ2d 1510, 1513 (Fed. Circ. 1993).

ii. Examiner's arguments

In the Final Office Action dated February 24, 2005, the Examiner alleges that the claims are not enabled because the specification does not provide any guidance because the function of the encoded polypeptide is, allegedly, unknown. The Examiner contends that other than the assertion that the polypeptide is a "putative" tumor suppressor, the specification provides no guidance regarding how the encoded polypeptide functions. The Examiner argues and that it would require undue experimentation as to which of the residues in the claimed sequences encompassed by the term "95% identity to SEQ ID NO:2" could be altered without changing critical function. The conclusion in the rejection is that one of skill would not know how to make or use the claimed invention.

iii. Claims 1, 30, 32, 33, 36, and 37 are enabled

As detailed below, the application provides ample guidance for making and using the claimed sequences that is commensurate to the scope of the claims. Although such analyses could conceivably require analyzing a large number of sequences, the practitioner could reasonably expect to be able to successfully identify sequences that fall within the scope of the invention and to use them in accordance with the disclosure in the specification. Further, the Examiner fails to establish a *prima facie* case that the claims are not enabled because the evidence provided in support of the arguments fails to properly support those arguments.

In the rejection, the Examiner contends that one of skill would not know how to make or use the claimed sequences because the function of the protein is not known. In particular, the Examiner alleges that Applicants have provided no guidance in terms of mutations that abolish critical functions or domains. These assertions, however, are unfounded. First, a biological role of the protein is in fact known: it plays a role in neoplastic disease, such as the disease multiple endocrine neoplasia type 1 (*see, e.g.,* page 1, lines 1-3). Applicants have shown numerous mutations in all of the *MEN1* coding exons (2, 3, 4, 5, 6, 7, 8, 9, and 10) that lead to nonfunctional *MEN1* alleles in patients having multiple endocrine neoplasia type 1 (*see, e.g.,* Figures 3 and 4, and page 52, lines 27 bridging to page 53, lines 12). In addition, three relatively common polymorphisms, one of which leads to a change in protein sequence, were identified that were present in normal chromosomes.

a. A practitioner in the art can identify members of the claimed genus.

The claims at issue in this rejections are directed to nucleic acid sequences that are characterized by the structural feature of encoding a polypeptide that shares a high percent identity to a reference sequence, SEQ ID NO:2. Although the claimed genus encompasses a large number of nucleic acids, the specification provides guidance such that one of skill in the art could identify any one of the claimed nucleic acids that fall within the scope of the claims. For example, the specification teaches how to isolate nucleic acid sequences that encode menin proteins (*see, e.g.,* page 18, lines 3-6). The application also teaches that a practitioner could

readily use manual or computer sequence alignments using SEQ ID NO:2 as a structural reference point to determine whether nucleic acid sequences have the specified identity (*see, e.g.*, page 14, lines 22-27; page 18, lines 21-27; and page 10, lines 26 through page 20, lines 26.)

Regarding the issue of enablement of nucleic acids where a large number of possible embodiments exist, the PTO has provided express guidelines for examination. As noted above, a rejection for undue breadth is inappropriate where one of skill could readily determine any one of the claimed embodiments (MPEP § 2164.08). In the present application, one of skill needs to identify nucleic acids that have a very high level of identity (at least 95%) with respect to a conserved reference sequence. Appellants have in fact done so, as shown in the examples. Additional evidence that the claims are enabled is provided in the form of a post-filing publication by Guru *et al., Mammalian Genom*e 10:592-596, 1999 (Appendix A in the Evidence Appendix; filed as part of Appellants' response filed January 13, 2003.) Guru *et al.* teach the isolation, genomic organization and expression of a mouse *MEN1* gene. The amino acid sequence encoded by the mouse gene has 97% identity to human menin, which has the sequence set froth in SEQ ID NO:2 (*see, e.g.,* Guru et *al.,* Figure 1, page 593). Thus, Appellants have provided ample evidence that although many nucleic acids encompassed by the genus are theoretically possible, one of skill can readily determine, one by one, any particular sequence that has these properties without undue experimentation.

b. The specification teaches how to use the claimed sequences.

In the Final Office Action mailed February 24, 2005, the Examiner argues that even if one of skill in the art were able to identify the claimed sequences, the practitioner would not be able to use the sequences, because, according to the Examiner, no function has been identified. The Examiner refers to a previous Office Action (May 25, 2003) in which publications by Bowie, Burgess, Lazar and Bork were cited. The cited art is described by the Examiner as teaching that one amino acid sequence can change function and that database searching by homology is a poor predictor of activity. The Examiner contends that these references thus provide evidence that one of skill in the art could not reasonably expect to

identify and use nucleic acids encoding polypeptides having at least 95% identity to SEQ ID NO:2 as menin proteins.

As previously noted, Appellants have, however, identified a biological function for *MEN1*. It plays a role in neoplastic disease such as the syndrome multiple endocrine neoplasia type 1. The references cited by the Examiner do not dispute this finding. The claimed sequences share a high degree of structural identity. The specification teaches that the nucleic acid sequences of the invention can be used in methods such as detecting the presence or absence of a *MEN1* gene (*see, e.g.,* page 34, lines 27-32) in a patient, or relative of a patient with multiple endocrine neoplasia type 1, or as a source of menin protein, *e.g.,* to produce antibodies for analyzing menin proteins in the patients. These teachings, in conjunction with the guidance provide for identifying members of the genus, therefore adequately enable the claims ("any enabled use that would reasonably correlate with the entire scope of that claim is sufficient to preclude a rejection for nonenablement based on how to use." MPEP, § 2164.01(c)). The Examiner provides no reasoning or evidence as to why one of skill could not reasonably expect the highly homologous structures as claimed to function similarly. Thus, the rejection does not establish a proper *prima facie* case that claims 1, 30, 32, 33, 36, and 37 are not enabled.

c. Claim 32 is additionally enabled.

Claim 32 is enabled for the reasons explained above and for additional reasons. Claim 32 relates to a transfected cell where the heterologous nucleic acid expressed by the cell comprises a nucleic acid as set forth in SEQ ID NO:1 or SEQ ID NO:3. These are specific nucleic acid sequences. Methods of transfecting cells are well known in the art (*see, e.g.,* the guidance in the specification provided in the sections beginning on page 25 relating to expression in prokaryotes and eukaryotes.) The specification additionally teaches how to use the claimed cells, *e.g.,* as a source for a probe to detect the presence or absence of a *MEN1* gene (*see, e.g.,* page 33, lines 27-29) or to express menin protein. Thus, the application explicitly teaches one of skill how to make and use nucleic acids comprising the specific sequences set forth in claim 32. The Examiner provides no evidence or reasoning to the contrary, other than the reasoning set

forth with regard to the separate rejection of claims 3, 4, and 32, which is addressed below. Accordingly, The Examiner has failed to establish a *prima facie* case that one of skill in the art could not make and use a cell as claimed.

d. Claims 36 and 37 are enabled

Claims 36 and 37 relate to expression cassettes and expression vectors. The Examiner appears to take the position that one of skill in the art must know a precise biological activity in order to express a protein. However, Appellants have provided evidence that one of skill in the art can readily identify nucleic acids that encodes variants of the exemplary menin protein shown in SEQ ID NO:2. This genus of variants as set forth in claims 36 and 37 all share a very high degree of sequence identity to SEQ ID NO:2 and can be used not only as a probe but for other applications, for example, as noted above to express menin proteins, *e.g.,* in order to make antibodies. The Examiner has provided no reasoning or evidence that such antibodies would not be useful to detect menin protein in patients with multiple endocrine neoplasia type 1. Accordingly, the Examiner has filed to establish a proper case that claims 36 and 37 are not enabled.

In view of the foregoing, Appellants respectfully request reversal of the rejection of claims 1, 30, 32, 33, 36, and 37 as not enabled.

B. Rejection of claim 1, 3-5, 19-24, 26, 30, 32, 33, 36, and 37

i. Examiner's arguments

The Examiner alleges that claims 1, 3-5, 19-24, 26, 30, 32, 33, 36, and 37 are additionally rejected as not enabled because one of skill would not know how to use menin proteins. The Examiner cites Wautot *et al. al., Int. J. Cancer* 85:877-881, 2000 (Appendix B of the Evidence Appendix; filed as part of Appellants' response filed January 13, 2003), as teaching that one of skill would not know how to use a protein encoded by the gene or antibodies specific for the encoded protein. In particular, the Examiner refers to page 880, column 2 of Wautot *et al.*, that refers to experiments in which no obvious alterations in menin expression levels and

cellular location were observed in lymphoblastoid cell lines developed from patients harboring a mutation in one of their MEN1 alleles. The Examiner further relies on Guru *et al., supra* as teaching that the putative tumor suppressor protein offers no clue to the function of the protein. The Examiner argues that in view of these teachings, one of skill would not know how to use menin proteins.

ii. Claims 1, 3-5, 19-24, 26, 30, 32,33, 36, and 37 are properly enabled.

Applicants first note that the claims at issued in this rejection are not directed to menin proteins. The claims are directed to nucleic acids that relate to the *MEN1* gene, which nucleic acids are characterized by their ability to encode menin proteins. An exemplary menin sequence is provided in SEQ ID NO:2. As previously explained, the *MEN1* gene has been shown to be mutated in patient with endocrine neoplasia. The claimed sequences therefore have been demonstrated to have a biological function. The references cited by the Examiner do not provide any evidence or suggestion to the contrary.

The Examiner appears to believe that because Wautot *et al.* did not note a difference in the levels of menin protein in lymphoblastoid cell lines developed from patients harboring a mutation in one of their MEN1 alleles, one of skill would not know how to use proteins or antibodies encoded by the claimed nucleic acid sequences. First, the significance of this observation in terms of the function is unclear, as the Examiner did not provide evidence or reasoning as to how this observation applies to the loss of *MEN1* function in neoplastic tissues such as endocrine tumors. (There is no disclosure that the lymphoblastoid cell lines are neoplastic.)

Second, the cited art does not provide any evidence that defects in *MEN1* genes are not believed to underlie the disease multiple endocrine type 1 neoplasia. To the contrary, Wautot *et al.* specifically states that the responsible gene has been identified (first paragraph of column 1, page 877). Accordingly, as asserted in the specification, one of skill could use antibodies to examine menin levels in patients with the disease for many purposes, including to further characterize the disease in any individual patient.

a. Claims 1, 3-5, 30, 32, and 33 are enabled for the reasons set forth in section VII(B) of this paper.

Claims 1, 3-5, 30, 32, 33, 36 and 37 are further enabled for the reasons explained above in section VII(B). Again, nucleic acids and host cells comprising the claimed nucleic acid can be used for diagnostic purposes. For example, they can be used as probes in southern blots. Furthermore, one of skill can use such sequences as controls to detect the presence or absence of *MEN1* sequences, or specific variants of *MEN1* (*see, e.g.,* page 36, lines 4-8). The compositions of claims 1, 3-5, 30, 32, 33, 36, and 37 are not limited by a recited use. Accordingly, as noted above, the rejection of the claimed compositions is improper (MPEP § 2164.01(c)).

b. Claims 36 and 37 are enabled for the reasons set forth in section VII(B) of this paper.

Claims 36 and 36 are additionally enabled for the reasons described above in section VII(B). The expression cassettes and vectors can be used as probes. Furthermore, Appellants have provided evidence that one of skill in the art can readily identify nucleic acids that encodes variants of the exemplary menin protein shown in SEQ ID NO:2. This genus of variants as set forth in claims 36 and 37 all share a very high degree of sequence identity to SEQ ID NO:2 and can be used, for example, as noted above to express menin proteins in order to make antibodies. Accordingly, the rejection is improper.

c. Claims 19-24 and 26 are additionally enabled.

Claim 19-24 and 26 relate to methods of detecting the presence or absence of a mutation in human *MEN1* gene encoding a human menin as set forth in SEQ ID NO:2 or the presence or absence of the gene. The citation of Wautot *et al.* and Guru *et al.* is irrelevant. Neither reference disputes the role of *MEN1* in the disease multiple endocrine neoplasia type 1. Accordingly, the Examiner has failed to provide proper reasoning or evidence that one of skill would not know how to make and use the claimed invention.

In view of the foregoing, Appellants respectfully request reversal of the rejection of claims 1, 3-5, 19-24, 26, 30, 32,33, 36, and 37 as allegedly lacking enablement.

C. Rejection of claims 3, 4, and 32 under 35 U.S.C. § 112

i. Examiner's arguments

The Examiner alleges that claims 3, 4, and 32, which relate to SEQ ID NOs. 1 and 3, are not enabled because of the open comprising terminology. Specifically, the Examiner contends that the claims read on the entire chromosome and that one of skill would not know how to use the sequences as claimed because one of skill would not know how to clone and express a chromosome in an expression vector, or how to use a chromosome as a probe.

ii. The Examiner has filed to establish a proper case that the claims are not enabled.

The Examiner's arguments rest on the assertion that the claims read on inoperative embodiments. However, the presence of inoperative embodiments within the scope of a claim does not necessarily render a claim nonenabled. The standard is whether a skilled person could determine which embodiments that were conceived, but not yet made, would be inoperative or operative with expenditure of no more effort that is normally required in the art. (MPEP § 2164.08(b), citing *Atlase Power Co. v. E.I. du Pont de Nemours $ Co.*, 750 F.2d 1569, 1577, 224 USPQ 409, 414 (Fed. Cir. 1984). The rejection fails, however, to establish that claims 3, 4, and 32 do not meet this standard. The Examiner provides no reasoning or evidence as to why one of skill would not be able to recognize working embodiments of the invention as claimed.

The Examiner speculates about embodiments without providing any evidence or reasoning that the postulated scenarios would be reasonable to one of skill in the art. For example, the Examiner speculates that a practitioner in the art would attempt to express menin protein by cloning a whole chromosomes into an expression vector to express menin protein (set forth on page 5 of the Final Office Action dated February 24, 2005). No evidence is provided

that one of skill could not reasonably determine whether or not this is a working embodiment. Thus, the Examiner fails to meet the burden of establishing a *prima facie* case of nonenablement.

a. Claim 3 is enabled.

Claim 3 recites an isolated or recombinant nucleic acid where the sequence comprises the coding region of SEQ ID NO:1. The Examiner argues that the chromosome comprises the coding region of SEQ ID NO:1 and that one of skill in the art would not know how to express the chromosome in an expression vector because of the stop codons or would not no how to use the whole chromosome as a probe. The Examiner's arguments are not well founded. SEQ ID NO:1 is a cDNA sequence (*e.g.*, page 10, lines 16-18), not a genomic sequence. As explained below, claim 3 relates to an isolated or recombinant nucleic acid sequence, not a whole chromosome. Even assuming *arguendo* that the claim did read on the whole chromosome, the chromosomal *MEN1* sequence includes introns. A cDNA sequence does not. Thus, the Examiner has failed to establish a *prima facie* case of nonenablement of this claim.

b. Claim 4 is enabled.

Claim 4 recites that the isolated or recombinant nucleic acid comprises SEQ ID NO:3. The Examiner again contends that this claim reads on an entire chromosome. The Examiner additionally alleges that one of skill would not be able to use the entire chromosome as a probe to evaluate *MEN1* DNA in a sample. Again, this argument is not well founded. As explained below with regard to the rejection under 35 U.S.C. § 102, claim 1 does not read on a whole chromosome. Even assuming *arguendo* that it did, this is an advanced art. The Examiner provides no evidence that one of skill in the art could not reasonably be expected to recognize workable (or non-workable) embodiments. Accordingly, the Examiner has failed to establish a *prima facie* case that claim 4 is not enabled.

c. Claim 32 is enabled.

Claim 32 recites a host cell where the heterologous nucleic acid comprises SEQ ID NO:1 or SEQ ID NO:3. The Examiner did not provide specific arguments supporting the contention that this claim was not enabled. The arguments appear to be based on the assertions that one of skill would not be able to express a whole chromosome in a host cell. Appellants traverse this rejection. Again, there is no evidence or reasoning that one of skill in the art would not be able to recognize the working embodiments postulated by the Examiner. Accordingly, the Examiner has not established a case that this claim is not enabled.

In view of the foregoing, it is respectfully requested that the rejection of claims 3, 4, and 32 be reversed.

D. Rejection of claims 1, 30, 32, 33, 36, and 37 under 35 U.S.C. § 112, first paragraph-written description

i. Standards for written description

Written description does not require that the disclosure as originally filed provide *in haec verba* support for claimed subject matter (*see, e.g., Purdue Pharma L.P. V. Faulding, Inc.* 230 F.3d 1320, 1323, 56 USPQ2d 1481, 1483 (Fed. Cir. 2000)). It is not necessary that the application describe the claim limitations exactly, but only to the extent that one of ordinary skill in the art would recognize from the disclosure that applicants invented the subject matter (*In re Herschler*, 591 F.2d 693, 200 USPQ 711 (CCPA 1979).

The Federal Circuit has expressly considered the application of the written description requirement to inventions in the field of biotechnology. See, *University of California v. Eli Lilly & Co.*, 119 F.3d 1559, 43 USPQ2d 1398 (Fed. Cir. 1997). The Court held that the written description requirement can be fulfilled in any number of ways, so long as the specification describes the invention in sufficient detail that one skilled in the art can clearly conclude that the inventor invented the claimed invention. For a chemical invention, an adequate description "requires a precise definition, such as by structure, formula, chemical name, or

physical properties...." (119 F.3d at 1568, 43 USPQ2d at1406.) The Court also addressed the manner by which a genus of cDNAs might be described, when it stated:

> A description of a genus of cDNAs may be achieved by means of a recitation of a representative number of cDNAs, defined by nucleotide sequence, falling within the scope of the genus or of a recitation of structural features common to the members of the genus, which features constitute a substantial portion of the genus. (also 119 F.3d at 1568, 43 USPQ2d at 1406).

Written description requirement in the context of DNA-related inventions has been further discussed by the Federal Circuit in *Enzo Biochem. Inc. v. Gen-Probe Inc.*, 296 F.3d 1316, 63 USPQ2d 1609 (Fed. Cir. 2002). In *Enzo*, the Court defined a standard where "the written description requirement can be met by 'showing that an invention is complete by disclosure of sufficiently detailed, relevant identifying characteristics... i.e., complete or partial structure, other physical and/or chemical properties, functional characteristics when coupled with a known or disclosed correlation between function and structure, or some combination of such characteristics." F.3d 1316 at 1324, 63 USPQ2d at 1613.

ii. Examiner's arguments

Claims 1, 30, 32, 33, 36, and 37 remain rejected as allegedly lacking adequate written description support. The Examiner contends that an isolated nucleic acid encoding a polypeptide having at least 95% identity to SEQ ID NO:2 is not supported by proper written descriptive support in the specification. Although Applicants have identified menin-encoding nucleic acids in the specification, *e.g.*, in Example 1 and Figures 3 and 4, the Examiner contends that there is no correlation between the structural hallmark set forth in the claims and function of the claimed genus.

iii. The claims meet the standard for written description.

As repeatedly noted, a biological role of the protein is in fact known: it plays a role in neoplastic disease such as the disease multiple endocrine neoplasia type 1 (*see, e.g.*, page 1, lines 1-3); thus the claimed sequences have a function. Further, the specification teaches

numerous mutations in all of the *MEN1* coding exons (2, 3, 4, 5, 6, 7, 8, 9, and 10) that lead to nonfunctional MEN1 alleles in patients having multiple endocrine neoplasia type 1 (*see, e.g.,* Figures 3 and 4, and page 52, lines 27 bridging to page 53, lines 12). The specification also discloses polymorphic variants, which are present in normal chromosomes, one of which results in a change in the protein sequence.

The claims are drawn to a genus of compositions that are highly homologous, nucleic acids that encode proteins comprising an amino acid sequence having at least 95% identity to SEQ ID NO:2. Thus, in contrast to *Lilly,* the claims provide a structural hallmark. The MPEP (§ 2163(II)(3)(a)), quoting *Lilly*, states that "[I]n claims involving chemical materials, generic formulae usually indicate with a specificity what the generic claims encompasses. One skilled in the art can distinguish such a formula from others and can identify many of the species that the claims encompass. Accordingly, such a formula is normally an adequate description of the claimed genus." Here, one of skill can identify the species encompassed by the claims. This meets the requirements explicitly state in *Lilly*.

With regard to the Examiner's allegation that there is no correlation between the structural hallmark and any function, as Applicants have pointed out, a structural element is set out in the instant claims, the reference sequence SEQ ID NO:2. There is in fact a correlation between this structural characteristic and a function: As indicated above, the specification demonstrates mutations in nucleic acids encoding SEQ ID NO:2 can lead to a disease, multiple endocrine neoplasia type 1.

As to the number of species that must be disclosed to enable a genus, as noted above, Appellants have provided structural properties that identify the species within the genus. Appellants have additionally described species, *e.g.,* nucleic acids that encode variants of SEQ ID NO:2 in the application. Thus, the disclosure of multiple species, in conjunction with the structural characteristics set forth in the claims is properly supported by the description in the specification.

a. Claims 32 and 33 are properly supported.

Claims 32 and 33 are additionally properly supported by description in the specification. Claims 32 and 33 recite particular SEQ ID NOs. (1 and 3) that are disclosed in the specification. Thus, the claims are fully described.

In view of the foregoing, Appellants request that the rejection of claims 1, 30, 32, 33, 36, and 37 as lacking proper written descriptive support be reversed.

E. Rejection of claims 19-24 and 26 under 35 U.S.C. § 112, first paragraph-written description

i. Examiner's arguments

Claims 19-24 and 26 were also rejected as allegedly lacking adequate written description support. These claims are drawn to methods (and kits) for detecting mutant and normal *MEN1* alleles, *i.e.*, mutant and normal genes that encode menin (SEQ ID NO:2). The Examiner contends that the specification teaches only a single gene/cDNA that is mutated in multiple endocrine neoplasia-type 1 and therefore does not provide adequate written description of polynucleotides that encode SEQ ID NO:2 that are mutated or deleted so that one would be able to predictably identify those included in the claimed genus.

ii. The specification describes mutant and normal *MEN1* alleles

Appellants have disclosed a wildtype *MEN1* allele and many mutated forms of *MEN1 (see, e.g.*, page 15, lines 13-17). Mutations occur in all of the coding exons (2-10). Furthermore, Applicants have identified polymorphism in the gene encoding SEQ ID NO:2 (*see, e.g.*, page 53, lines 4-6). Applicants have provided exemplary primers and probes that can be used to detect mutant and normal *MEN1* alleles (*see, e.g.*, exemplary primers and probes in Table 1 on page 18).

Further, claims 19-24 and 26 recite a structural hallmark, the reference sequence, SEQ ID NO:2. The MPEP (§ 2163 III)(3))a)(ii))unequivocally states that

> in the molecular biology arts, if an applicant disclosed an amino acid sequence, it would be unnecessary to provide an explicit disclosure of nucleic acid sequence that encoded the amino acid sequence. Since the genetic code is widely known, a disclosure of an amino acid sequence would provide sufficient information such that one would accept that an applicant was in possession of the full genus of nucleic acids encoding a give amino acid sequence.".

In view of the foregoing, the use of SEQ ID NO:2 proper describes multiple nucleic acids. In conjunction with the disclosure of exemplary mutations *MEN1* genes in patients, the specification supports the claims in compliance with the standards for written description. Appellants therefore respectfully request reversal of this rejection.

F. Rejection of claims 1, 3-5, 30, 32, 33, 36, and 37 under 35 U.S.C. § 102(b)

i. Anticipation standard

To anticipate a pending claim, a prior art reference must provide, either expressly or implicitly, each and every limitation of the pending claim. MPEP §2131.

ii. Examiner's arguments

Claims 1, 3-5, 30, 32, 36, and 37 were rejected as allegedly anticipated. The Examiner contends that the term "isolated" as defined in the application encompasses human chromosome 11 present in a somatic cell hybrid panel disclosed in U.S. Patent No. 4,594,318. On page 10, first full paragraph of the final Office Action mailed February 24, 2005, the Examiner specifically alleges that the cited patent teaches an isolated human chromosome 11, and a transfected cell comprising the chromosome, which, according to the Examiner, is defined by the specification as an expression vector. The Examiner suggests that the expression vector comprises the entire chromosome.

iii. The cited art does not disclose each and every limitation of the claims

a. Claims 1, 4, 5, and 30

The Examiner argues that the presence of the human chromosome in the somatic cell hybrid panel is anticipatory in view or the definition of "isolated" provided in the specification. However, this analysis discounts the complete definition of "isolated" provided in the specification. In particular, the Examiner failed to consider the definition provided in the context of a gene. In the definition of "isolated" on page 10 (continued from page 9) of the specification, it is explicitly states that "[i]n particular, an isolated *MEN1* gene is separated from open reading frames which flank the gene and encode a protein other than the *MEN1* gene product." Thus, the specification teaches that "isolated" in context of a genomic *MEN1* nucleic acid sequence encompassed by claims 1, 4, and 5 refers to a sequence that is separated from the open reading frame that flank the gene and encode a different protein. U.S. Patent No. 4,594,318 does not teach this element. Accordingly, the reference is not anticipatory.

b. Rejection of claim 3 and claim 32 to the extent that it relates to SEQ ID NO:1

As noted above, SEQ ID NO:1 is a cDNA sequence. Claim 3 relates to an isolated or recombinant sequence that comprises the coding region of SEQ ID NO:1. Chromosome 11 does not contain an isolated cDNA sequence. Accordingly, claim 3 is also additionally free of the art.

c. Claims 36 and 37

The Examiner is apparently alleging that the somatic cell hybrid line CHO-K1 described in U.S. Patent No. 4,594,318 is an expression vector. However, claims 36 and 37 also relate to an expression cassette. The specification teaches that an expression cassette refers to a recombinant expression system for expressing a nucleic acid sequence of the invention (*see, e.g.*, page 9, lines 12-20.). U.S. Patent No. 4,594,318 does not disclose that the somatic cell hybrid

line CHO-K1 is a recombinant expression system. Accordingly, the reference does not anticipate claims 36 and 37.

In view of the foregoing, reversal of the rejection of claims 1, 3-5, 30, 32, 33, 36, and 37 is respectfully requested.

G. Rejection of claim under 35 U.S.C. § 112, second paragraph

i. Standard for definiteness

According to the MPEP §2173, to satisfy the statutory requirement under 35 U.S.C. §112, second paragraph, claims must particularly point out and distinctly claim the subject matter of the invention. The essential inquiry pertaining to the definiteness requirement is whether a claim sets out and circumscribes a particular subject matter with a reasonable degree of clarity and particularity so as to appraise one of skill in the art of the claim scope. An indefiniteness rejection is appropriate when a person of ordinary skill in the art could not interpret the metes and bounds of the claim. MPEP §2173.02.

ii. Examiner's arguments

The Examiner contends that the recitation of a "a transfected cell *in vitro*" in claim 30 is indefinite because it is not clear whether the claim is drawn to a cell transfected *in vitro* or whether the claims is drawn to a cell that was transfected in any manner and is now found *in vitro*.

iii. Claim 30 is definite

The examiner's focus during examination of claims for compliance with the requirement for definiteness is whether the claim meets the threshold requirements of clarity and precision, not whether more suitable language or modes of expression are available (MPEP § 2173.02)). Claim 30 clearly refers to a transfected cell, where the cell is *in vitro*, as evidenced by the grammar (it does not recite "an *in vitro*-transfected cell"). Thus, the claim does not read on a cell that is present in a subject, *e.g.*, a human. In the Final Office Action of February 24, 2005,

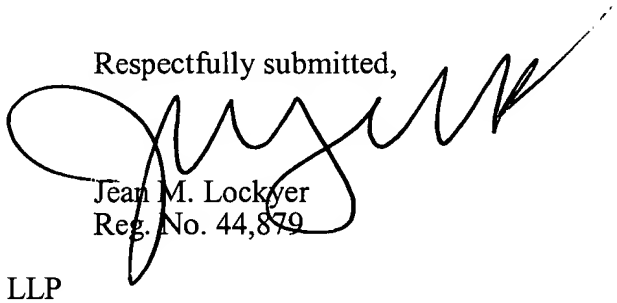
the Examiner noted that the rejection would be obviated by amending the claim to read "an isolated transfected cell". It is not clear how the recommended claim language is distinguishable from the current recitation of "a transfected *in vitro*", as the end result would be the same, *i.e.*, such an amendment would avoid reading on a cell *in vivo*. Accordingly, the current claim language obviates the rejection in the same manner that the suggested claim language obviates the rejection. Appellants therefore respectfully request reversal of this rejection.

H. Conclusion

For all of the above reasons, the claims are compliant with the standards for patentability. It is respectfully requested that all of the outstanding rejections be reversed.

Appellants believe no additional fees are due in view of the payment of the requisite fee pursuant to 37 CFR §1.17(f) for the Appeal Brief filed March 1, 2006. However, if any additional fees are associated with this substitute Brief, please charge deposit account 20-1430.

Respectfully submitted,



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VIII. CLAIMS APPENDIX

Claims involved in the appeal:

1. (previously presented) An isolated or recombinant nucleic acid encoding menin, wherein said nucleic acid encodes a protein comprising an amino acid sequence having at least 95% identity to SEQ ID NO:2.

2. (cancelled)

3. (previously presented) The isolated or recombinant nucleic acid of claim 1, wherein the sequence comprises the coding region of SEQ ID NO:1.

4. (previously presented) The isolated or recombinant nucleic acid of claim1, wherein the sequence comprises SEQ ID NO:3.

5. (previously presented) The isolated or recombinant nucleic acid of claim 1, wherein the nucleic acid sequence encodes a protein having the sequence set forth in SEQ ID NO:2.

6.-18. (cancelled)

19. (previously presented) A method for detecting in a test sample the presence or absence of a mutation in a human MEN1 gene comprising a nucleotide sequence that encodes a human menin as set forth in SEQ ID NO:2, or the presence or absence of the MEN1 gene, the method comprising:

a) contacting said test sample with a first oligonucleotide having a sequence that discriminates between a wild type gene and the missing allele or mutant form; and,

b) detecting the formation of a duplex between the gene and the first oligonucleotide sequence.

20. (previously presented) A method of claim 19, wherein the first oligonucleotide is unable to bind to the wild-type MEN1 gene under hybridization conditions in which the first oligonucleotide binds to the mutant sequence of MEN1.

21. (original) A method of claim 19, wherein the contacting step further comprises amplifying a portion of the human MEN1 gene and where the first nucleic acid is a polymerase chain reaction amplification primer which binds to an intron of MEN1.

22. (original) A method of claim 19, wherein the contacting step further comprises amplifying a portion of MEN1 and where the first nucleic acid is a polymerase chain reaction amplification primer which discriminates between wild-type and mutant forms of MEN1 using allelic specific polymerase chain reaction.

23. (original) A method of claim 19, wherein the first nucleic acid binds to either exons or introns of the genomic DNA encoding the human menin gene.

24. (previously presented) A kit for detecting in a test sample the presence or absence of a mutation in a MEN1 gene comprising a nucleotide sequence encoding a menin polypeptide as set forth in SEQ ID NO:2, the kit comprising;

a) a container holding a first oligonucleotide sequence that discriminates between the wild type gene and the mutant form; and

b) a container holding a reagent for detecting the formation of a duplex between the gene and the first nucleotide sequence.

25. (cancelled)

26. (previously presented) The kit of claim 24, further comprising amplification primer pairs specifically binding to a human genomic DNA sequence encoding menin.

27.-29. (cancelled)

30. (previously presented) A transfected cell *in vitro*, wherein the cell comprises a heterologous nucleic acid of claim 1.

31. (cancelled)

32. (previously presented) The transfected cell of claim 30, wherein the heterologous nucleic acid comprises a nucleic acid as set forth in SEQ ID NO:1 or SEQ ID NO:3.

33. (previously presented) The transfected cell of claim 30, wherein the cell is a human cell.

34. (withdrawn) An organism into which an exogenous nucleic acid sequence has been introduced, the exogenous nucleic acid specifically hybridizing under stringent conditions to a nucleic acid with:

a sequence as set forth in SEQ ID NO:1; or,

a nucleic acid encoding a protein defined as having a calculated molecular weight of about 67.5 kDa; and (a) specifically binding to an antibody raised against an protein with a sequence as set forth in SEQ ID NO:2; or (b) having at least 60% amino acid sequence identity to a protein with a sequence as set forth in SEQ ID NO:2; and,

the organism expresses the exogenous nucleic acid as a menin protein.

35. (withdrawn) The organism of claim 34, wherein the exogenous nucleic acid comprises the nucleic acid as set forth in SEQ ID NO:1 or SEQ ID NO:3.

36. (previously presented) An expression cassette comprising a nucleic acid of claim 1, wherein the nucleic acid is operably linked to a promoter.

37. (original) The expression cassette of claim 36, further comprising an expression vector.

38.-42. (cancelled)

IX. EVIDENCE APPENDIX

A. Guru *et al., Mammalian Genome* 10:592-596, 1999

a) filed as an appendix with Appellants' amendment filed January 13, 2003

b) The Office Action mailed March 25, 2003 acknowledged that the amendment had been entered.

B. Wautot *et al., Int. J. Cancer* 85:877-881, 2000

a) filed as an appendix with Appellants' amendment filed January 13, 2003

b) The Office Action mailed March 25, 2003 acknowledged that the amendment had been entered.

Mammalian Genome 10, 592–596 (1999).

Mammalian Genome
Incorporating Mouse Genome

Isolation, genomic organization, and expression analysis of *Men1*, the murine homolog of the MEN1 gene

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Abstract. The mouse homolog of the human MEN1 gene, which is defective in a dominant familial cancer syndrome, multiple endocrine neoplasia type 1 (MEN1), has been identified and characterized. The mouse *Men1* transcript contains an open reading frame encoding a protein of 611 amino acids which has 97% identity and 98% similarity to human menin. Sequence of the entire *Men1* gene (9.3 kb) was assembled, revealing 10 exons, with exon 1 being non-coding; a polymorphic tetranucleotide repeat was located in the 5′- flanking region. The exon-intron organization and the size of the coding exons 2–9 were well conserved between the human and mouse genes. Fluorescence in situ hybridization localized the *Men1* gene to mouse Chromosome (Chr) 19, a region known to be syntenic to human Chr 11q13, the locus for the MEN1 gene. Northern analysis indicated two messages—2.7 kb and 3.1 kb—expressed in all stages of the embryo analyzed and in all eight adult tissues tested. The larger transcript differs from the smaller by the inclusion of an unspliced intron 1. Whole-mount in situ hybridization of 10.5-day and 11.5-day embryos showed ubiquitous expression of *Men1* RNA. Western analysis with antibodies raised against a conserved C-terminal peptide identified an approximately 67-kDa protein in the lysates of adult mouse brain, kidney, liver, pancreas, and spleen tissues, consistent with the size of human menin. The levels of mouse menin do not appear to fluctuate during the cell cycle.

Introduction

Multiple endocrine neoplasia type 1 (MEN1) is an autosomal dominant familial cancer syndrome characterized by tumors of the parathyroids, enteropancreatic endocrine tissues, and the anterior pituitary (Metz et al. 1994). The MEN1 locus was mapped to human Chr 11q13 by linkage analysis (Larsson et al. 1988), and the responsible MEN1 gene was identified by positional cloning (Chandrasekharappa et al. 1997). The human MEN1 gene is organized into 10 exons (the first being untranslated) and is ubiquitously expressed as a 2.8-kb transcript. This transcript encodes a 610-amino acid product, termed menin, which exhibits no apparent similarities to any known proteins. Over 200 independent germline and somatic mutations spread throughout the coding region of menin have been described (Marx et al. 1999). Somatic mutations are observed to a variable extent in certain sporadic tumors: parathyroid adenoma, gastrinoma, insulinoma, and lung carcinoid (Marx et al. 1999). MEN1 appears to be a typical tumor suppressor gene: lack of menin owing to inactivation of both alleles probably leads to the development of tumors.

Although no murine syndrome similar to MEN1 has been reported to date, here we detail the identification of the mouse MEN1 homologous gene, *Men1*, on mouse Chr 19. The cDNA sequence, genomic organization, RNA and protein expression patterns are presented and discussed.

Materials and methods

Screening BAC library. The entire Men1 cDNA sequence was assembled by sequencing the insert in a cDNA clone (IMAGE clone ID 557658). DNA pools from a BAC library prepared from 129Sv/cJ7 mouse DNA (Research Genetics, Huntsville, AL) were screened by PCR with primers (5′-GCTGAAGGCGCCCAGAAGACG-3′ and 5′-CTGAGCGGTGAATCGGGCATAGAG-3′) designed from the mouse *Men1* cDNA sequence. Three BAC clones, 331J21, 331K21, and 7D23, were isolated. The size of the inserts was determined by pulsed-field gel electrophoresis of *Not*I-digested BAC DNAs as described earlier (Guru et al. 1997b).

Subcloning and sequence analysis. DNA isolated from the BAC clone 7D23 was subjected to partial digestion with *Sau*3A1 to generate fragments of approx. 10–12 kb. The fragments were cloned into the *Bam*H1 site of the plasmid pBluescriptII KS+ (pBSIIKS+). Colonies were analyzed by PCR with STSs designed for both the 5′- and 3′-ends of the *Men1* cDNA in order to identify the clones containing the entire gene. One clone with an approximate 10-kb insert containing the entire *Men1* gene was chosen for subsequent sequence analysis and genomic characterization.

Primers, UP1 (GACATCCATGGCTACACAGAAAAACCC) and LP1 (GCCTGTGTAAAGGGAAGAAGACAGAGAGAGT), generating a 260-bp product, were used for the PCR amplification of the $(AAAG)_{10}$ repeat alleles from mouse genomic DNA.

Northern analysis. A 900-bp insert representing the 3′-end of the *Men1* cDNA clone (IMAGE clone ID. 402210) was released by digestion of the plasmid DNA with *Eco*RI and *Not*I, labeled with a random primer labeling kit (Amersham) and hybridized to a mouse multiple tissue Northern blot (#7762-1) and embryo blot (#7763-1) (Clontech, Palo Alto, CA) as described earlier (Guru et al. 1997a). For the 5′-end probe, a 299-bp PCR product representing most of intron 1 was amplified from the BAC clone 331J21.

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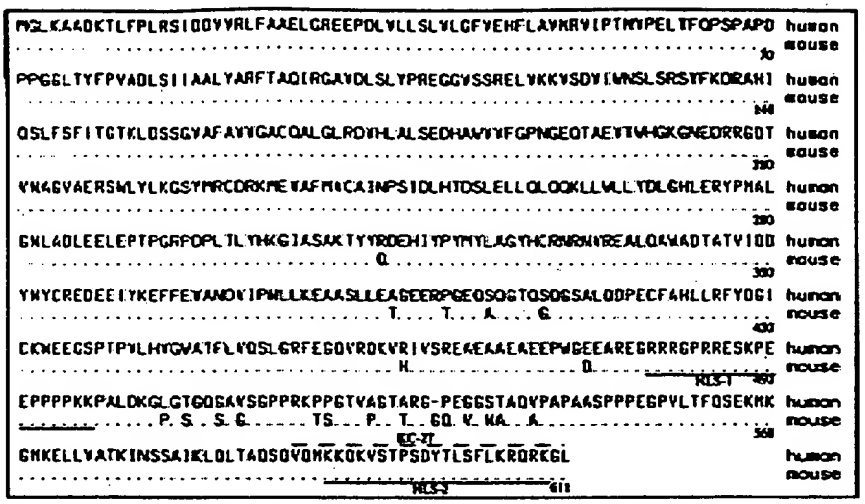

Fig. 1. a) Comparison of human and mouse menin sequences. Alignment of the amino acid sequence was carried out using Multiple Sequence Alignment program of DNASTAR. The human and mouse menin sequences are shown on the top and bottom respectively. An identical amino acid in mouse menin is indicated by a dot. The numbering of amino acid refers to the mouse protein sequence. The C-terminal 27 aa sequence used for producing a rabbit antibody (KC-27) is indicated with a broken line on top of the sequence. The two nuclear localization signals (NLS-1 and NLS-2) mapped in human menin are underlined. b) Western analysis of protein lysates from five adult mouse tissues and HeLa cells (human) probed with KC27 antibodies. The location of menin is indicated on the left, and those of size markers on the right.

Western analysis. Mouse tissues were homogenized in Tris-HCl buffer [50 mM Tris-HCl pH 8.0, 100 mM NaCl, 0.3 mM DTT, 10 mM $MgCl_2$, 10% glycerol, 0.5% NP-40, 100 μg/ml AEBSF [4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride], 1 μg/ml aprotinin/leupeptin] and centrifuged at 20,000 *g* for 1 h. The supernatant was mixed with Laemmli buffer, boiled for 5 min, and then the proteins (40 μg for each tissue and 10 μg for HeLa cell lysate) were separated on a 10–20% SDS gel. Production of KC27 antibodies in rabbits to a C-terminal human menin 27 aa peptide and routine Western analysis procedures were as described previously (Guru et al. 1998).

Fluorescence in situ hybridization (FISH). Preparation of metaphase chromosomes from lymphocytes obtained from mouse spleen, hybridization conditions, and analysis of fluorescent signals were as described previously (Pecker et al. 1996). The BAC clone (331J21) containing the mouse *Men1* gene was labeled by nick translation with biotin-conjugated dUTP. In order to facilitate chromosome identification, a digoxigenin-labeled mouse Chr 19-specific painting probe was used for co-hybridization. The biotinylated probes were detected by incubation with avidin-FITC, and the digoxigenin sequences were detected with mouse anti-digoxin and goat anti-mouse conjugated to TRITC (Sigma Chemical Co., St. Louis, Mo.). Chromosomes were counterstained with DAPI.

Whole-mount mouse embryo in situ hybridization. Non-radioactive in situ hybridization was performed on whole embryos [10.5 and 11.5 days post coitum (p.c.)] and cryosections (12.5 days p.c.) prepared as previously described (Kos et al. 1999). Embryos were obtained from matings of FVB mice. Noon of the plug day was considered 0.5 days p.c., but embryonic ages were confirmed by comparison of somite number and external features with descriptions by Kaufman (1992). Sense and antisense digoxigenin-labeled riboprobes were generated from linearized templates by in vitro transcription with T3 or T7 RNA polymerase. A plasmid (IMAGE clone ID. 402210) with an insert representing the 900-bp 3′-end of the *Men1* cDNA was used as a template. For antisense probe, the plasmid was linearized with *Eco*RI and transcribed with T3 RNA polymerase. For sense probe (negative control), the plasmid was linearized with *Not*I and transcribed with T7 RNA polymerase.

Cell synchronization. Synchronization of cells, preparation of protein lysates, and Western analysis were as described earlier (Brown et al. 1997). Briefly, NIH-3T3 cells were synchronized in G_0 by culturing the cells for 96 h under reduced serum [0.5% fetal calf serum (FCS)] conditions in Dulbecco's Modified Eagle Medium (DMEM). To collect cells synchronized in G_1, serum-starved cells were replated in complete growth medium (DMEM supplemented with 10% FCS) for 6 h. Mitotic (M) cells were obtained by culturing in complete growth medium supplemented with colcemid (0.1 μg/ml) for 24 h, and mitotic cells were collected by mitotic shake-off. Extracts of NIH-3T3 cells in log phase growth (Log) were obtained from asynchronous cultures grown in complete growth medium. Aliquots (1×10^6 cells) of asynchronous and synchronized cell populations were stained with propidium idodide and analyzed by flow cytometry to confirm their stage in cell cycle.

Results

Mouse Men1 cDNA sequence and its encoded 611 aa mouse menin protein. A search of the NCBI EST database (dbEST) with human MEN1 cDNA sequence revealed the availability of several mouse cDNA clones with similar sequences. Analysis of the homologous regions in the available EST sequences and the sizes of the clone inserts resulted in identification of the longest cDNA clone (IMAGE clone ID. 557658). A 2984-bp sequence (Genbank acc. no. AF109389) was assembled for the entire cDNA insert in this clone. The sequence includes an ORF encoding the putative mouse menin protein of 611 aa, one amino acid longer than that of human menin (610 aa). The encoded mouse protein shows 97% identity

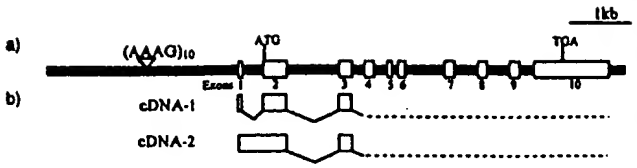

Fig. 2. a) Exon–intron organization of the *Men1* gene. The 9330-bp genomic sequence is shown as a thick horizontal line, and the locations of the 10 exons (open boxes), the tetranucleotide repeat $(AAAG)_{10}$ in the 5′-flanking region, the initiation codon ATG in exon 2, and the termination codon (TGA) in exon 10 are shown. The exon numbers are indicated below. b) A diagram depicting the differences near the 5′-end of the two types of transcripts: cDNA-1, completely spliced 2592-bp transcript. cDNA-2, a 2984-bp transcript with unspliced intron 1. The dotted line indicates that there are no changes in the remaining part of the two transcripts.

with and 98% similarity to human menin sequence (Fig. 1a). Antibodies (KC27) raised against a conserved C-terminal peptide (indicated in Fig. 1a) were used for the identification of mouse menin by Western analysis of lysates from adult mouse brain, kidney, liver, pancreas, and spleen tissues. All the tissues showed a protein of approx. 67 kDa, similar in size to that of human menin (Guru et al. 1998) from HeLa cell lysate (Fig. 1b).

Men1 gene sequence and exon-intron organization. Three BAC clones were isolated by screening a mouse BAC library with an STS specific for the Men1 cDNA sequence. The inserts in clones 331J21 and 331K21 were similar in size (280 kb), whereas that of the clone 7D23 was 120 kb. In addition to their similar size, the identical *Not*I enzyme restriction pattern and their adjacency in the library suggested that the clones 331J21 and 331K21 are likely to be copies. PCR analysis with primers amplifying both ends of the mouse Men1 cDNA sequence revealed that all BAC clones had the entire *Men1* gene.

In order to obtain genomic sequence of the *Men1* gene, a plasmid library was prepared by cloning *Sau*3A1 digests of the BAC clone 7D23 into the *Bam*H1 site of pBSIIKS+. PCR analysis of plasmid clones identified a clone with an approximate 10-kb insert containing the entire *Men1* gene, and a 9286-bp sequence (Genbank acc. no. AF109390) of the mouse gene was assembled by sequencing this insert. The genomic sequence revealed that, similar to the human gene, the mouse gene also consists of 10 exons, and the locations of the initiation (ATG) and the termination codons are in exons 2 and 10, respectively (Fig. 2a). The sizes of the intervening exons 2–9 are identical in human and mouse. Comparison of homologous mouse ESTs in the dbEST database to the assembled Men1 genomic and full-length cDNA sequences indicated two types of cDNA clones. One resembled the human, and transcripts with an unspliced intron 1 (362 bp) along with the otherwise fully processed transcripts contributed the second type of mouse Men1 cDNA clones (Fig. 2b). However, the 611-aa ORF with the translation initiation point (ATG) in exon 2 remained unaltered in both types of messages. A single variant polyadenylation signal (GATAAA) is located 20 nucleotides upstream of the poly-A stretch in all seven Men1 cDNA clones analyzed.

A tetranucleotide repeat $(AAAG)_{10}$ was observed about 1.5 kb upstream of the 1st exon. PCR amplification of the repeat region in DNA samples from various strains of mice revealed four different alleles indicating that this repeat is polymorphic. Allele sizes in *DBA/2J, FVB/NJ,* and *M. spretus* are different from each other. The $(AAAG)_{10}$ allele was observed in C57bl/6J, A/J, and 129/SvJ mice (data not shown).

Chromosomal localization of the Men1 gene by FISH. Chromosomal localization of the *Men1* gene was carried out by FISH analysis of mouse metaphase chromosomes. A biotin-labeled

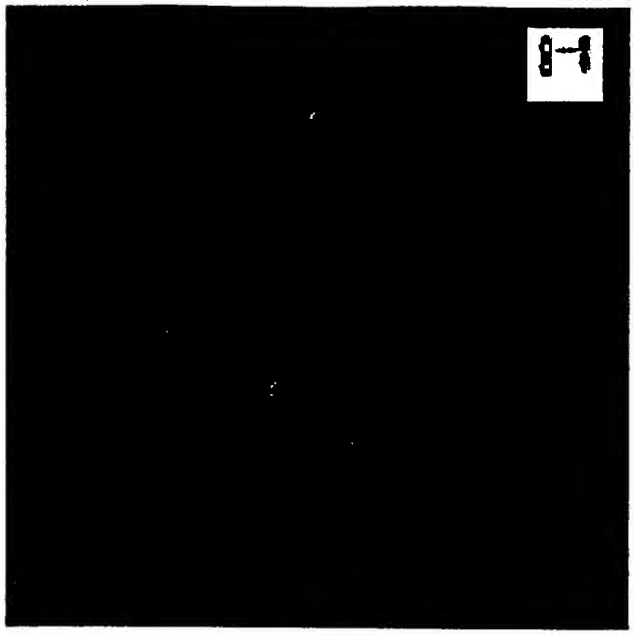

Fig. 3. Fluorescent in situ hybridization of *Men1* gene to mouse metaphase chromosomes. Labeled BAC clone 331J21 DNA (green signal) and Chr 19-specific painting probes (red signal) were cohybridized to mouse metaphase chromosomes. The location of the green signal owing to hybridization of *Men1* containing BAC DNA on the proximal region of Chr 19, between cytogenetic bands B and C2, is depicted in the inset.

331J21 BAC DNA probe was cohybridized with a digoxigenin-labeled mouse Chr 19-specific painting probe. The BAC clone (green signal) hybridized to the proximal region of mouse Chr 19 (red signal; Fig. 3). This region Chr 19 is syntenic with human pericentric Chr 11q13 containing the MEN1 gene. The BAC clone hybridization signals were observed exclusively at Chr 19.

Men1 gene expression by Northern analysis. A multiple-tissue Northern blot representing RNA from eight adult mouse tissues (Clontech) was probed with a labeled 900-bp cDNA insert representing the 3′-end of the Men1 cDNA. The presence of two messages, sized at 2.7 kb and 3.1 kb, was observed in all tissues, although the extent of expression between the tissues varied considerably (Fig. 4a). The expressions in heart, spleen, and skeletal muscle were reduced compared with brain, lung, liver, kidney, and testis. Both messages appear to be expressed in nearly equal amounts in all tissues except for brain, where the longer transcript was prominent. In order to resolve the nature of the two messages, a 299-bp probe generated by PCR amplification of most of intron 1 was hybridized to the same Northern blot. Only the longer 3.1-kb message hybridized to the intron 1 probe, indicating that the longer message originated owing to an unspliced intron 1 (Fig. 4b).

Analysis of *Men1* gene expression during embryonic development with a Northern blot with RNAs from 7-, 11-, 15-, and 17-day embryos showed that both messages (2.7 kb and 3.1 kb) were expressed at all these stages (Fig. 4c). Minimal variation was observed as to the relative amounts of the two messages.

Whole-mount in situ hybridization. In situ hybridization was used to examine the distribution of Men1 mRNA during mouse embryogenesis. Wild-type embryos were examined by whole-mount in situ hybridization at 10.5 and 11.5 days p.c. and by hybridization to cryosections at 12.5 days p.c. At all ages examined, Men1 mRNA appeared to be ubiquitously expressed throghout the embryos. Hybridization result from the 11.5 day p.c. embryo is shown in Fig. 5. Examination of cross sections indicated a higher level of

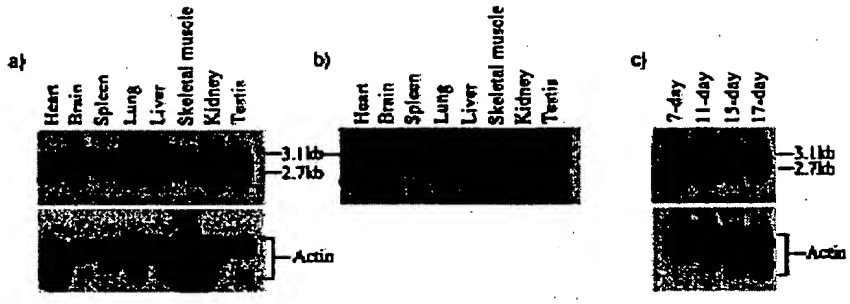

Fig. 4. *Men1*, gene expression by Northern blot analysis. **a)** A multiple-tissue Northern blot (Clontech) with RNA from eight adult mouse tissues (indicated on the top) was hybridized to a 900-bp radiolabeled probe representing the 3′-end of the *Men1* cDNA. Size and location of the longer (3.1 kb) and the smaller (2.7 kb) messages are indicated. The signals obtained from a beta-actin probe used as a control for RNA loading on this blot are shown at the bottom. **b)** The same blot was probed with a radiolabeled 299-bp probe representing intron 1. Hybridization of this probe only to the longer 3.1 kb message is indicated. **c)** Hybridization of a Northern blot containing RNA from 7-, 11-, 15- and 17-day mouse embryos with the 900-bp Men1 cDNA probe as in (a). The locations of the 3.1-kb and 2.7-kb messages are shown. Hybridization of this blot to a control beta-actin probe is shown below.

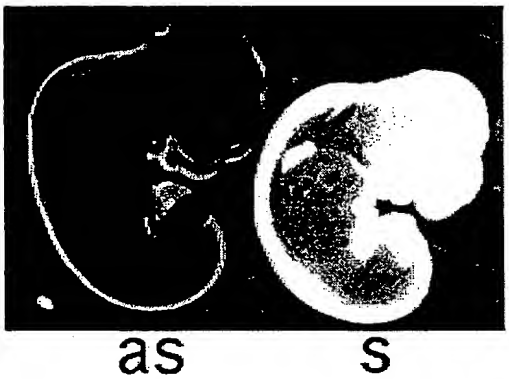

Fig. 5. Expression of *Men1* in mouse embryos by whole-mount in situ hybridization. Antisense (as) and sense (s; control) RNA probes generated by T7 and T3 RNA polymerase with a cDNA clone representing the 900 bp from the 3′-end of Men1 cDNA were hybridized to an 11.5-day embryo.

expression in cranial ganglia, sensory ganglia, and neural tube than observed in adjacent tissues.

Mouse menin expression in the cell cycle. Cultures of the murine cell line NIH-3T3 were synchronized at various points in the cell cycle (G_0, G_1, and M phase) and, along with asynchronous cultures of cells in log-phase growth (Log), were analyzed by Western analysis with KC27 antibodies. The menin levels in all these lysates were equivalent, indicating that menin expression does not fluctuate during the cell cycle.

Discussion

The mouse homolog (*Men1*) of the human MEN1 gene, located as expected at the syntenic region on Chr 19, has been isolated and characterized. A similar exon-intron structure, identical sizes of coding exons, 88% similarity of the coding nucleotide sequence, and 97% identity of the encoded proteins indicate that the *Men1* gene is highly conserved through evolution. In addition to an extra amino acid in the mouse menin, there are only 20 locations where the amino acids differ in the entire length of the two proteins (Fig. 1a). It is interesting to note that none of the 46 amino acids involved in disease-associated germline and/or somatic missense mutations or inframe deletions are different in mouse menin, showing the importance of conservation in defining the structural and functional role of menin. The two nuclear localization signals (Guru et al. 1998) have been completely conserved in mouse (Fig. 1a). Therefore, mouse menin is also expected to be predominantly located in the nucleus.

The sequence of the entire mouse *Men1* gene (9.3 kb) is also reported here (AF109390). A polymorphic tetranucleotide repeat present in the 5′-flank of the *Men1* gene may be a helpful genetic marker in looking for loss of heterozygosity in mouse tumors in which this gene might play a role. While this manuscript was in preparation and revision, two other groups independently reported an analysis of the mouse *Men1* gene (Stewart et al. 1998; Bassett et al. 1999). Our results are in agreement with these two reports. The overlapping sequences of Men1 reported here (Genbank acc. no. AF109389) and those of Stewart and associates (Genbank acc. no. AF016398) are identical, but differ from the sequences reported by Bassett and coworkers (Genbank acc. no. AF072755) at three locations. These differences are: at codons 457 (I changing to M), 466 (E changing to G), and 512 (S changing to L). In these three positions, the sequences reported here are conserved and identical to that of the human menin sequence. The sequences we assembled from seven independent cDNA clones for the 3′ end indicate a GATAAA sequence, located 19 nt upstream of the polyA stretch, as the likely polyadenylation signal.

The mouse *Men1* gene is expressed in at least two alternate forms—an additional isoform arises owing to alternative splicing of intron 1. Both types of messages appear to be expressed nearly equally. The ORF and, therefore, the sequence of the encoded mouse menin are unaltered in these two messages, but alternative translational efficiency is possible.

Northern analysis indicates widespread expression of *Men1* in all embryonic stages (7-day to 17-day) and in all eight adult tissues tested. The significance of the modest expression level differences between different tissues and at different embryonic stages, if any, is unclear.

The distribution of Men1 mRNA was also examined by whole mount in situ hybridization. In mouse embryos, *Men1* was ubiquitously expressed, with somewhat higher levels in neural tissues. The ubiquitous expression and early embryonic expression of mouse menin is suggestive of its fundamental role in a widespread biological function. The molecular basis for the development of neoplasia in restricted endocrine tissues in patients with MEN1, despite ubiquitous expression of the gene, remains to be determined. It is possible that other genes are able to compensate for lost MEN1 function in the unaffected tissues. Analysis of organ function in mice with engineered alterations in *Men1* will be useful for understanding the role of *Men1* in development and disease.

Antibodies raised against a C-terminal peptide that is identical in human and mouse allowed identification of mouse menin by Western analysis (Fig. 1b). The size of the mouse protein, as expected, is similar to that of the human, and levels of menin do not appear to fluctuate during the cell cycle (Fig. 6). These anti-

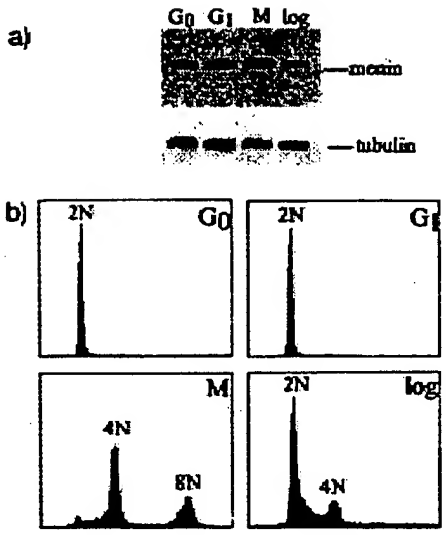

Fig. 6. Mouse menin levels in NIH-3T3 cells at different stages of cell cycle. a) Using the KC27 antibody, menin levels were analyzed by Western blot (whole cell extract; 10 μg protein each lane) from extracts derived from NIH-3T3 cells synchronized in the G_0, G_1, and Mitotic (M) phases of the cell cycle, as well as cells from asynchronous cultures in log phase of the cell growth (Log). The same blot was probed with a tubulin antibody to confirm that equivalent amounts of protein were present in each lane. b) Aliquots of cells were stained with propidium iodide and subjected to flow cytometric analysis. Shown are histograms where cell number is plotted on the Y-axis and DNA content on the X-axis, confirming the cell cycle stage of the synchronized cell populations analyzed in a.

bodies may be useful in future biochemical characterization of mouse menin.

This characterization of the mouse *Men1* gene and its protein product should now set the stage for development of a mouse model of the human disease.

Acknowledgments. We thank Sunita Agarwal for critical reading of the manuscript.

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EXPRESSION ANALYSIS OF ENDOGENOUS MENIN, THE PRODUCT OF THE MULTIPLE ENDOCRINE NEOPLASIA TYPE 1 GENE, IN CELL LINES AND HUMAN TISSUES

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We have investigated the endogenous expression of menin, a protein encoded by the gene mutated in multiple endocrine neoplasia type 1 (MEN1). Western blot analysis showed strong expression of menin as a 68 kDa protein in all of 7 human and primate cell lines tested. In a panel of 12 fetal human tissue extracts, 68 kDa menin was readily detected in brain cortex, kidney, pituitary, testis and thymus and weakly detected in thyroid. Reproducible bands other than 68 kDa were observed in adrenal and heart, whereas menin was undetectable in liver, lung, pancreas and skin. Analysis of synchronized HeLa cells revealed no variation in the amount or size of menin throughout the cell cycle. Protein expression was compared between lymphoblastoid cell lines from healthy controls and MEN1 patients carrying nonsense mutations on 1 allele. No truncated protein was detected in either cytoplasmic or nuclear fractions in mutation-carrying cells. The expression level and cellular location of full-length menin did not differ between cell lines derived from MEN1 patients and healthy donors. This suggests that the wild-type allele has been up-regulated in mutation-carrying cells to compensate for the loss of 1 functional allele. *Int. J. Cancer* 85: 877–881, 2000.
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Multiple endocrine neoplasia type 1 (MEN1) (MIM: 131100) is a hereditary syndrome transmitted as an autosomal dominant trait. It is characterized by multiple occurrence of tumors of the parathyroids, endocrine pancreas and anterior pituitary (Wermer, 1954). Other endocrine neoplasms, such as adrenal tumors and carcinoids, and non-endocrine tumors, such as lipoma, are seen less frequently (Trump *et al.*, 1996). The disease locus was previously mapped to 11q13 near the PYGM marker (Larsson *et al.*, 1988), and the responsible gene has been identified (Chandrasekharappa *et al.*, 1997; European Consortium on MEN1, 1997). The *MEN1* gene covers a 9 kb genomic region and produces a widely expressed transcript of 3 kb. The open reading frame (ORF) is composed of 610 codons and is translated into a 68 kDa protein named menin. A direct interaction between menin and JunD has been demonstrated, and expression of wild-type menin can inhibit the transcriptional activity of JunD *in vitro* (Agarwal *et al.*, 1999).

To date, the identified *MEN1* mutations are mainly frameshift or nonsense mutations, which coincide with loss of heterozygosity in both familial and sporadic MEN1-associated tumors (Agarwal *et al.*, 1997; Bassett *et al.*, 1998; Giraud *et al.*, 1998). These patterns of inactivation strongly suggest that menin acts as a tumor suppressor. Known missense mutations are scattered over the entire coding region, which suggests that multiple domains of the protein are responsible for its biological function.

It has been shown, by both immuno-histochemical and subcellular fractionation methods, that menin is located mainly in the nucleus (Guru *et al.*, 1998). Two putative nuclear localization signals (NLSs) have been found in the C-terminal region by deletion mapping. About 80% of the known frameshift and nonsense mutations lead to a truncated peptide lacking both NLS1 and NLS2 motifs, whereas NLS2 is maintained in 20% of mutations. Although NLS1 alone appears to be sufficient for the nuclear localization of the GFP–menin fusion protein *in vitro*, little is known about the functionality of these NLSs *in vivo* or the effect of mutations on the cellular location.

Here, we report the detection of endogenously expressed menin in a panel of both human and primate cell lines and 12 human embryonic tissues at week 20 of gestation. We further investigate the expression and subcellular localization of menin during the cell cycle and the effect of truncating mutations on menin in lymphoblastoid cell lines from MEN1 patients.

MATERIAL AND METHODS

Cell culture, cell transfection and tissues

Adherent and non-adherent cells were maintained under standard conditions in DMEM and RPMI-1640, respectively, using 10% FCS. Cell lines used in our study include Cos-M6, Bosc 23 [a highly transfectable ectopic virus-packaging cell line (Pear *et al.*, 1993)], GDH (a glioma line), HeLa, PC12, U87 [a glioma line (Furnari *et al.*, 1997)] and 4 lymphoblastoid cell lines (LCLs) previously established in our laboratory (Giraud *et al.*, 1998). The lines LCL-F23M1 and LCL-F42M1 carry germ-line mutations R415X and R527X, respectively, while LCL-FN1 and LCL-FN2 are from non-affected individuals from a MEN1 family. Transfections were performed by calcium phosphate co-precipitation according to standard protocols with the following modifications: 24 hr before transfection, Bosc cells were plated at 7×10^6 cells/ml per culture in a 100 mm dish and 30 μg of plasmid DNA used. The efficiency of transfection was increased by treatment with chloroquine at 25 μM. A panel of 12 normal human tissues were obtained from aborted fetuses of gestation week 20, including adrenal, brain cortex, heart, kidney, liver, lung, pancreas, pituitary, skin, testis, thymus and thyroid.

Cell-cycle synchronization

HeLa cells were serum-starved (0.5% FCS) for 48 hr to obtain cells at G_0 phase and treated by double thymidine block with 25 mM thymidine (Sigma-Aldrich, Saint Quentin Fallavier, France) for 16 hr, with an 8 hr release interval, and tested at 0, 3 and 6 hr, to obtain cells synchronized, respectively, at the G_1/S, S and S/G_2 boundaries. To obtain mitotic cells, HeLa cells were directly treated with 400 ng/ml nocodazol (Sigma-Aldrich) and incubated for 16 hr. Synchronized cells were harvested, washed 3 times with cold culture medium and subjected to subcellular fractionation as described below. Flow-cytometric analysis was done using FACSCALIBUR 3CA (Becton Dickinson, Mountain View, CA).

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Constructs

MEN1 cDNA was obtained from a human testis library (Stratagene, Saint Quentin en Yvelines, France). A tag sequence for the VSV-G epitope (YTDIEMNRLGK) was incorporated by PCR amplification of the 5′ end of the cDNA, using primers 5′-CCCCGCGGTATGTACACTGATATCGAAATGAACCGCCTGGGTAAGGGGCTGAAGGCCGCCCA-3′ (cDNA position 111–130), and 5′-CCGTTACGGGAACACCATCTC-3′ (position 921–943). The complete cDNA encoding the N-tagged menin was subcloned into a NotI-cut pCI-neo expression vector (Promega, Charbonnières, France) and referred to as pCI-NtM1-S. The constructs for menin expression in bacteria were assembled by inserting a PCR-generated *MEN1* cDNA (position 146–2050) into SmaI-cut pQE30-32 vector (Qiagen, Courtaboeuf, France). An in-frame construct, pQE-FM1, was used; an out-of-frame insert, pQE-FM4, was used as a negative control.

Protein extract preparation and isolation of nuclei

Extracts from cell lines were prepared as follows: the medium was removed and cell layers were rinsed with PBS and lysed in a modified RIPA lysis buffer containing NaCl 150 mM, EDTA 0.2 mM, Tris-HCl (pH 7.5) 20 mM, NP40 1% and SDS 0.2% supplemented with a cocktail of protease inhibitors (1 mM PMSF; 10 μg/ml aprotinin, leupeptin and pepstatin; 1 mM orthovanadate). After 20 min incubation at 4°C, the lysate was centrifuged at 13,000 *g* for 15 min at 4°C to remove insoluble material. Frozen tissues were pulverized and lysed in the above solution. Protein concentration was determined using a modified Bradford analysis kit (Bio-Rad, Ivry sur Seine, France).

Cellular cytoplasmic and nuclear fractions were separated using lysis in Nonidet P-40 buffer. Cell pellets were suspended in a 0.2 M sucrose/3 mM $CaCl_2$/2 mM magnesium acetate/0.1 mM EDTA/10 mM Tris-HCl (pH 7.5)/1% Nonidet P-40 buffer and incubated at 4°C for 30 min. After microscopic examination to ensure that cell lysis occurred and that the nuclei appeared to remain intact, the nuclear pellet was sedimented. The cytoplasmic fraction was centrifuged to remove any trace of nuclei and the nuclear pellet suspended in the modified RIPA buffer. Subcellular nuclear and cytoplasmic fractionations were monitored, respectively, using the antibody p300(N-15) against p300 human nuclear CREB co-activators (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-β-tubulin (Boehringer, Meylan, France). The absence of nuclear fraction leakage into the cytoplasmic fraction was further confirmed using an immunoblot for the BRCA1 protein with anti-BRCA1 antibody OP92 (Calbiochem, Meudon, France).

Generation of antibodies for menin detection

Polyclonal anti-menin antibodies were raised in rabbits against 2 synthesized peptides, composed of 14 and 23 amino acid peptides coupled with KHL, positioned at amino acids 388–401 for C15G and 588–610 for M23C, respectively. Both anti-sera were affinity-purified with the peptides used for immunization, then tested for menin detection. Primary antibodies were diluted at 1:1,000 for C15G, 1:5,000 for M23C, 1:5,000 for unpurified preimmune sera and 0.1 μg/ml for anti-VSV-G.

Western blot analysis

SDS-PAGE was performed as described (Sambrook *et al.*, 1989). Proteins transferred to PVDF membranes were stained against a 1% Ponceau S. solution (Sigma-Aldrich), to check loadings, and incubated with primary antibodies overnight at 4°C in a solution containing PBS, 0.1% Tween 20 and 4% milk. After washing with PBS plus 0.1% Tween 20 and incubation with conjugated secondary antibody, filters were incubated with enhanced chemiluminescent (ECL) substrate (Amersham, Les Ulis, France) and exposed to Hyperfilm (Amersham). For quantitative analysis of immunoblots using M23C, the chemiluminescence was captured and evaluated by an image analyzer (Image Reader LAS-1000; Fuji, Tokyo, Japan).

RESULTS

Generation and characterization of anti-menin antibodies

To analyze endogenous menin in cultured cells and human tissues, we have generated polyclonal antibodies directed against synthetic peptides, located at amino acids 388–401 for antibody C15G and at amino acids 588–610 for M23C. In Western blot analysis, both C15G and M23C recognized the menin fusion protein produced from the pQE-FM1 construct, while the control pQE-FM4 reacted negatively (Fig. 1*a*). A band of a similar size was also detected by the 2 antibodies in protein extracts from cell lines (Fig. 1*b*). The size of the endogenously expressed menin was further compared with that of the tagged menin expressed in Bosc cells transfected by pCI-NtM1-S. A doublet of 68 to 70 kDa was observed in Bosc cells transfected with the construct, whereas only a single band at 68 kDa was detected in non-transfected cells and cells transfected with vector only (Fig. 1*b*). The upper band was also detected, with an antibody directed against the VSV-G epitope used as a tag (Fig. 1*c*). Extracts from testis tissue and non-transfected Bosc cells gave the same signal at 68 kDa (Fig. 1*d*).

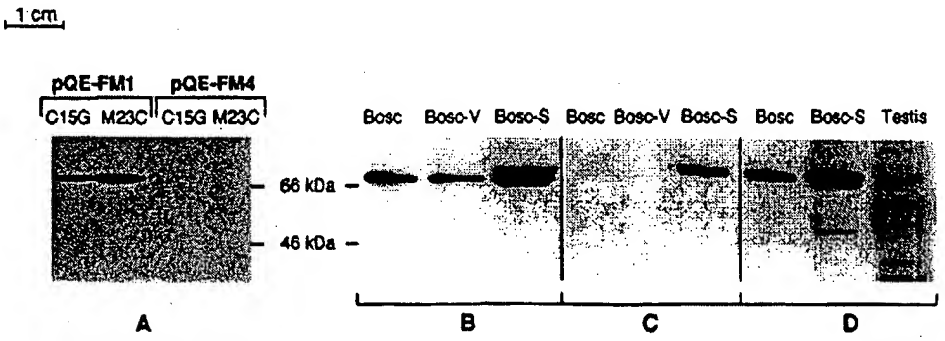


FIGURE 1 – Characterization of anti-menin antibodies. Authentication of anti-menin antibodies using a menin fusion protein *(a)*. The anti-menin antibodies used in our study, C15G and M23C, were reacted against a menin fusion protein, pQE–FM1, as well as negative control, pQE–FM4. Comparison of the gel-migration profile between endogenously expressed menin and exogenously tagged menin by immunoblot *(b–d)*. Protein (20 μg) was extracted from Bosc cells without or with vector only (Bosc-V); vectors containing tagged MEN1 cDNA in the sense direction (Bosc-S) were loaded and separated on a 10% SDS-PAGE gel. Immunoblots were first reacted with C15G *(b)* and, after stripping, with anti-VSV-G antibody *(c)*. The same amount of protein extracts from Bosc cells with or without vector containing tagged MEN1 cDNA in the sense direction and 30 μg of extracts from testis were tested using the above conditions with C15G *(d)*.

Detection of endogenously expressed menin in a panel of normal human tissues and in different cell lines

A panel of 12 normal human tissues from aborted fetuses at about 20 weeks of gestation was used for the detection of endogenous menin expression. A strong, 68 kDa, band was apparent in brain, pituitary, testis, thymus and kidney, while in thyroid only a weak band was detected (Fig. 2*a*). Several bands of other sizes, including a 40 kDa polypeptide seen in adrenal, heart and testis and a band of about 100 kDa in adrenal, were detected with both C15G and M23C; none of these bands was visualized with pre-immune sera (Fig. 2*b*). No bands were visible in liver, lung, pancreas and skin, even when 30 μg of protein extract from pancreas and skin were applied (Fig. 2*c*).

The cell lines selected for menin detection by Western blot using C15G and M23C antibodies were Bosc 23, Cos-M6, GDH, HeLa, PC12, U87 and 2 LCLs (LCL-FN1 and LCL-FN2) established from the non-affected individuals of a MEN1 family. Both C15G and M23C gave similar results. As shown in Figure 2*d*, one major band at 68 kDa was detected in all cell lines tested. An additional band at 40 kDa was visible in HeLa and U87 cells. None of the above bands was detected with pre-immune sera (data not shown).

Menin expression during the cell cycle

We investigated whether expression of menin was subject to any alteration during the cell cycle. For this purpose, HeLa cells were synchronized; harvested at time points corresponding to G_0/G_1, G_1/S, S, S/G_2 and M phases; and subsequently fractionated into cytoplasmic and nuclear extracts. Menin was apparently identical with the band detected on whole-cell extracts (Fig. 1*b*) in both nucleus and cytoplasm, though the cytoplasmic signal was weaker (Fig. 3*a*). As analyzed by autoradiography (Fig. 3*a*) and by image analysis of the chemiluminescence (data not shown), the level of menin remained approximately constant and without size alteration throughout the different cell cycle stages. A similar expression profile was obtained using synchronized NIH 3T3 cells (data not shown). The synchronization of HeLa cells was confirmed by FACS analysis (Fig. 3*c*). As a control for cell synchronization and subcellular fractionation, protein extracts were tested on immunoblot using the anti-BRCA1 antibody OP92. The variation of BRCA1 protein expression in the nucleus during cell-cycle progression (Fig. 3*b*) was as described previously (Chen *et al.*, 1996; Vaughn *et al.*, 1996).

Menin expression in LCLs carrying germ-line mutations

Two LCLs, carrying R415X and R527X germ-line mutations, respectively, were compared with 2 LCLs from non-affected individuals from a MEN1 family. Protein produced from the mutated alleles would lack both NLSs in the R415X and NLS2 in the R527X mutation. A strong signal at 68 kDa was observed in nuclear extracts of all 4 lines tested and a clearly weaker one in the cytoplasmic fractions (Fig. 4). No truncated peptide was detected with either C15G (data not shown) or M23C antibody. Expression of full-length menin was quantified as above and appeared at the same level compared with LCLs from non-affected individuals (Fig. 4).

DISCUSSION

Endogenous menin was detected in all human cell lines tested, whereas its expression appears to be more restricted in tissues. Our data are consistent with the results of transcriptional analysis using *in situ* RNA hybridization of the mouse embryos, in which a restricted transcription pattern was demonstrated at late gestational

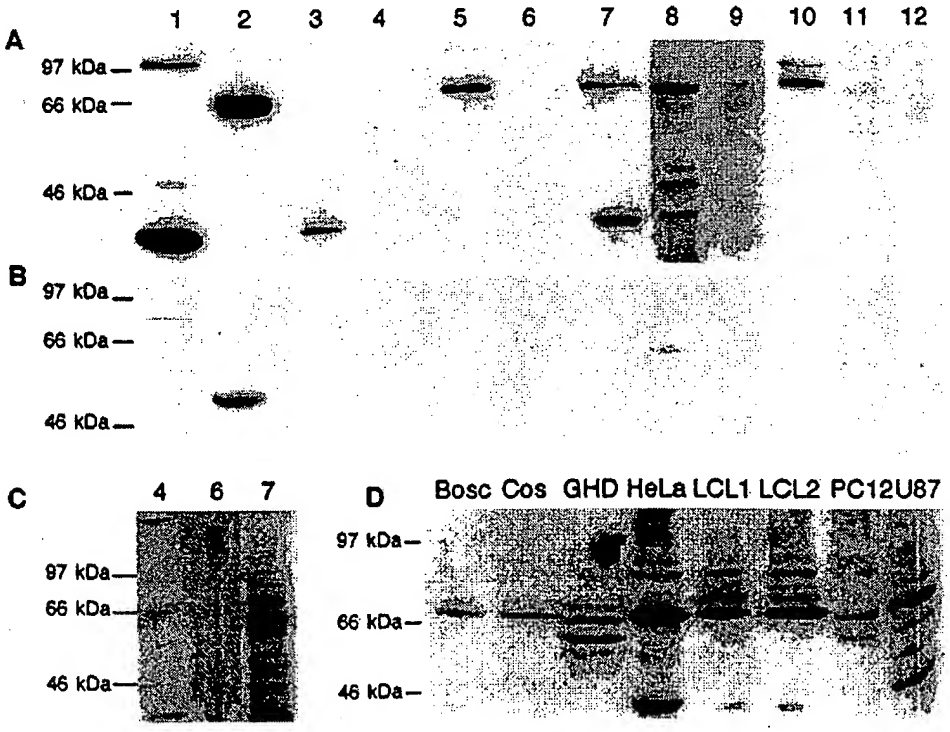
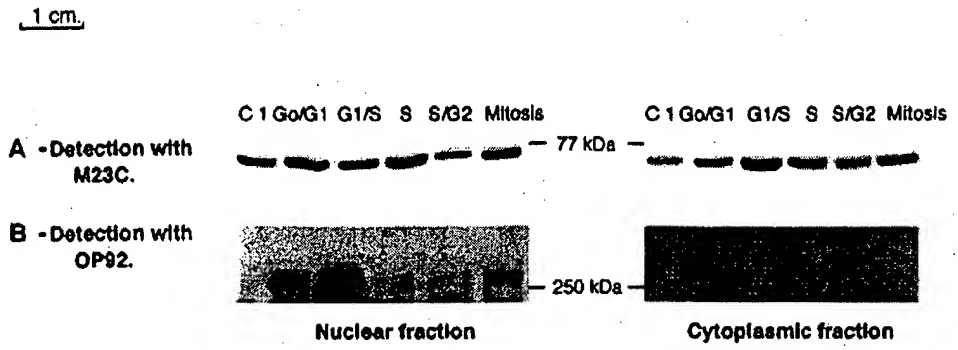


FIGURE 2 – Detection of endogenously expressed menin in normal human embryonic tissues and different cell lines by immunoblotting. For endogenous menin detection, 20 μg of protein from each of 12 human embryonic tissues at 20 weeks were used, including (1) adrenal, (2) brain cortex, (3) heart, (4) pancreas, (5) pituitary, (6) skin, (7) testis, (8) thymus, (9) thyroid, (10) kidney, (11) lung and (12) liver *(a,b)*. From pancreas and skin, 30 μg of protein were used to confirm the absence of menin in the above result *(c)*. Protein extracts (10 μg) from each of 8 cell lines *(d)* were also tested, including Bosc 23, Cos-M6, GHD, HeLa, LCL-FN1, LCL-FN2, PC12 and U87. Protein extracts were loaded and separated on a 10% SDS-PAGE gel for tissues or an 8% gel for cells and reacted to anti-menin serum *(a,c,d)* or to pre-immune sera *(b)*.



C - FACS Analysis

	C1	Starved (G0/G1)	T-0H (G1/S)	T-3H (S)	T-6H (S/G2)	Nocodazol (Mitosis)
G0/G1	60.2	70.0	40.8	26.9	20.9	31.5
S	19.2	20.3	36.5	55.9	33.1	18.4
G2/M	14.4	12.4	17.6	15.2	42.8	43.6

FIGURE 3 – Menin expression and its cellular location during cell-cycle progression. Cytoplasmic and nuclear protein extracts (10 μg) from synchronized HeLa cells at different stages were loaded on an 8% SDS-PAGE gel for menin detection by immunoblot with M23C *(a)*. An equal amount of the same extracts was loaded on a 6% SDS-PAGE gel and probed with an anti-BRCA1 serum, OP92 *(b)*. Markers used are RPN756 (Amersham), for detection with C15G and cytoplasmic fraction with OP92, and RPN800 (Amersham), for detection of nuclear fraction with OP92. C1 is protein extract control from the cells without treatment. The data obtained from FACS analysis of synchronized cells *(c)* show the percentage of distribution of cells in the various stages of the cell cycle.

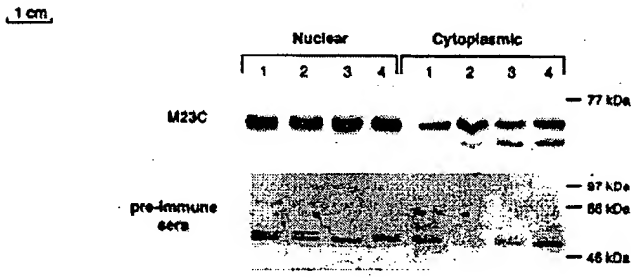


FIGURE 4 – Determination of the subcellular location of endogenous menin expressed in normal LCLs and LCLs carrying germ-line mutations removing NLS. Cytoplasmic and nuclear subcellular fractions were obtained from 2 LCLs established from the non-affected individuals of a MEN1 family (lanes 3 and 4) and 2 LCLs carrying germ-line mutations R415X (lane 2) and R527X (lane 1). Extracts (20 μg) from each line were loaded and migrated on a 10% SDS-PAGE gel. Immunoblots with the same loadings were reacted with M23C and pre-immune serum.

age (Stewart *et al.*, 1998). Expression analysis using multi-tissue Northern blots has indicated ubiquitous expression (European Consortium on MEN1, 1997), and menin protein has been detected in adult mouse pancreas (Guru *et al.*, 1999). It is possible that menin expression depends on the developmental stage, as with the *BRCA1* gene, whose expression decreases during mammary gland formation (Magdinier *et al.*, 1999). Fetal tissue samples were from gestational week 20, when the endocrine pancreas reaches the end stage of formation (Hoet *et al.*, 1995). It is also possible that the low level of endocrine cells in the fetal pancreas prevented menin from being successfully detected.

Menin is present at relatively constant levels throughout the cell cycle without undergoing any size alteration, in agreement with the data reported on NIH 3T3 cells (Guru *et al.*, 1999). This is in contrast with many proteins of the cell cycle, such as cyclins. Cell cycle–dependent post-translational modifications are, however, not excluded. Huang *et al.* (1999) have shown, by immunohistochemistry, that menin is localized mainly in the cytoplasm at M phase, as opposed to mainly in the nucleus during the other stages. We did not detect an M phase-specific increase of menin expression by Western blotting with either of our antibodies, one of which (C15G) was similar to that used by Huang *et al.* (1999). Since we used a different detection method, we do not exclude the possibility that the discrepancy is due to variation in signals other than the 68 kDa menin protein that we observed by Western blots.

We did not detect any obvious alteration in menin expression levels and cellular location in LCLs carrying germ-line mutations compared with LCLs from non-affected individuals. No truncated protein was detected in extracts from 2 LCLs carrying nonsense mutations, suggesting a fast degradation of non-functional protein. It appears that regulatory mechanisms maintain a constant level of expression, whether both alleles are functional or not. Interestingly, in sporadic parathyroid tumors, the loss of 1 allele did not result in reduced transcriptional levels (Farnebo *et al.*, 1998). Previous cytogenetic analyses have shown increased chromosomal instability in cell lines from MEN1 patients (Tomassetti *et al.*, 1995). This indicates that up-regulation of the wild-type allele may not fully compensate for loss of the other allele. The exact nature of these degradation and compensatory pathways of menin remains to be elucidated.

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